TAIBAH UNIVERSITY

COLLEGE OF PHARMACY

BIOCHEMISTRY II (PHT 383)

PRACTICAL PART

FIRST TERM

1443/2021

BLOOD

BLOOD

- Blood is the vehicle of transportation, and it makes up about 6% of total body weight. Blood is a liquid consisted of yellowish fluid called plasma in which cellular constituents are suspended.

- The cellular constituents of blood include red blood cells (erythrocytes), a variety of white blood cells (leukocytes) and platelets. Five classes of leukocytes (based on their morphologic characteristics on staining with Wright-Romanowski stain) are recognized: Neutrophils, Eosinophils, Basophils, Monocytes and lymphocytes.

- Neutrophils + Eosinophils + Basophils are called Granulocytes.

Plasma composition can be summarized as follows:

1) 90% water.

2) 10% solids:

- **7% plasma proteins.**
- **2% organic constituents other than plasma proteins.**
- **1% inorganic constituents = electrolytes e.g. Na^+, K^+, HCO_3^- , Cl^-, ... etc.**

A- Plasma proteins

The total protein concentration in plasma ranges between 7-8 gm %. Fractionation and separation of plasma proteins can be carried out by various methods including electrophoresis, precipitation at different salt concentrations, and chromatography.

Plasma proteins include:

1) **Albumins (3.5 - 4.5 gm %). They have the lowest molecular weight of the plasma proteins (69000 Da) and formed mainly in the liver.**

2) **Globulins (2.5-3 gm %). These have a molecular weight between 90,000 and 1000,000 Da and could be separated into sub-fractions. They include alpha, beta and gamma globulins.**

3) **Fibrinogen and prothrombin. Fibrinogen and prothrombin are formed in the liver. They are the precursors of fibrin and thrombin consequently.**

4) **Enzymes. Different enzymes are found in plasma. Plasma enzymes could be divided into two groups:**

   ***Group 1*: are specific to plasma e.g. lipoprotein lipase and the blood clotting enzyme.**
   ***Group 2*: are derived from other tissues e.g. transaminases (AST & ALT).**

   Changes in the levels of the second group occur in various diseases and thus they have an important diagnostic value.

5) **Transport proteins. Examples: lipoproteins, transferin and ceruloplasmin.**

Functions of Plasma Proteins:

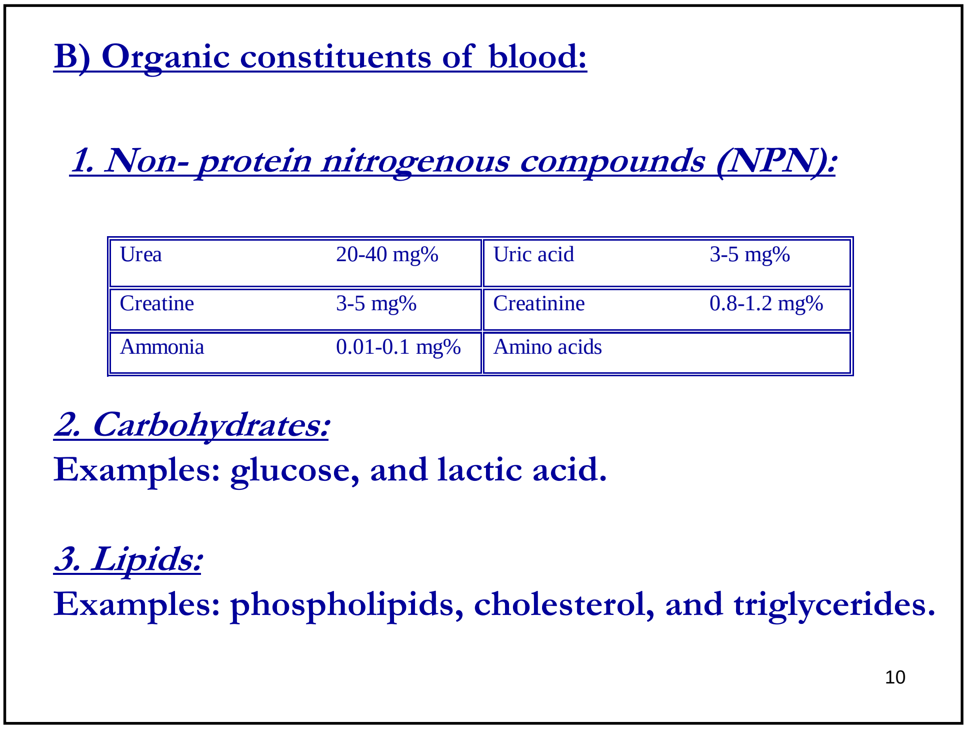
1) Maintenance of osmotic pressure of blood which is essential to keep normal blood volume.

2) Fibrinogen and prothrombin are essential for blood clotting.

3) Buffering action: Plasma proteins, like other proteins, are amphoteric and can combine with acids or bases, i.e. can act as buffers, and thus will share in maintenance of normal blood pH.

4) Protective function: Gamma globulins (immunoglobulins) form the antibodies that protect against diseases.

5) Transport function: Blood lipids which circulate in blood in the form of plasma lipoproteins. Iron which circulate in blood in the form of transferrin. Copper which circulate in blood in the form of ceruloplasmin. Thyroid hormones which circulate in blood in the form of protein bound iodine (PBI).



B) Organic constituents of blood:

1. Non- protein nitrogenous compounds (NPN):

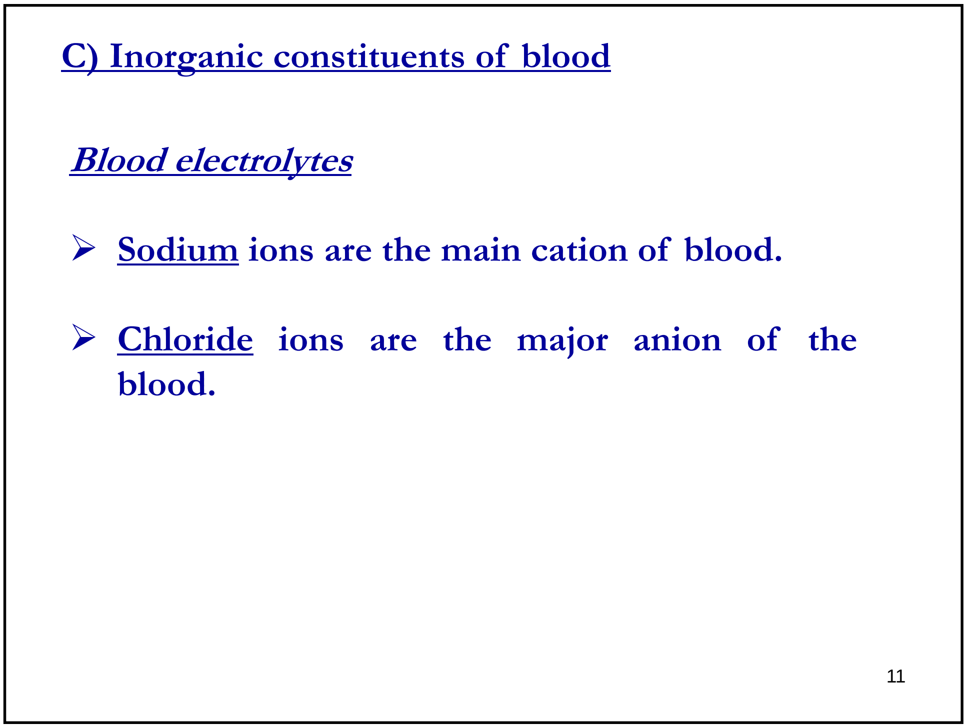
Urea	20-40 mg%	Uric acid	3-5 mg%
Creatine	3-5 mg%	Creatinine	0.8-1.2 mg%
Ammonia	0.01-0.1 mg%	Amino acids	

2. Carbohydrates:

Examples: glucose, and lactic acid.

3. Lipids:

Examples: phospholipids, cholesterol, and triglycerides.



C) Inorganic constituents of blood

Blood electrolytes

- **Sodium ions are the main cation of blood.**
- **Chloride ions are the major anion of the blood.**

- *When whole blood, containing an anti-coagulant, is centrifuged, the formed elements of blood sediment into a volume equal to 45% of the total volume of blood. The supernatant is known as plasma.*

- *On the other hand, if blood is allowed to clot and the clot is retracted. The fluid left is known as serum.*

Serum is plasma without the protein fibrinogen and other clotting factors.

Functions of blood

1) Respiration: transport of oxygen from the lungs to the tissues and of CO_2 from the tissues to the lungs.

2) Nutrition: transport of absorbed food materials.

3) Excretion: transport of metabolic waste to the kidneys, lungs, skin, and intestines for removal.

4) Maintenance of normal acid-base balance.

5) Regulation of body temperature by the distribution of body heat.

6) Defense against infection by the white blood cells and circulating antibodies.
7) Transport of signals, such as hormones for metabolism regulation.
8) Coagulation.

Blood buffers and acid base balance

- The normal blood pH is 7.4.
- The blood pH is kept at its normal levels due to the presence of potent and effective blood buffers which resist any changes in pH.

Blood buffers are either present in plasma and extracellular fluids or inside the RBCs. In plasma, the carbonic acid/bicarbonate buffer is the most important buffering mechanism(H_2CO_3 and $NaHCO_3$).

SPECTROPHOTOMETRY

Principles of spectrophotometry

- Many substances absorb light in the visible or ultraviolet region of the spectrum. This property can be used to determine the concentration of such a substance.

- The extent of light absorption depends on the type and concentration of the substance and on the wavelength of the light used.

- Monochromatic light: The light with a defined wavelength isolated from white light. Monochromator is therefore used.

The apparatus used in spectrophotometric analysis is called UV-visible spectrophotometer and its wavelength (λ) is ranged from 200 nm to 750 nm which depends on the light Source used:

1. **Tungsten lamp for visible region (400-750 nm).**
2. **Hydrogen discharge lamp for UV region (200-400 nm).**

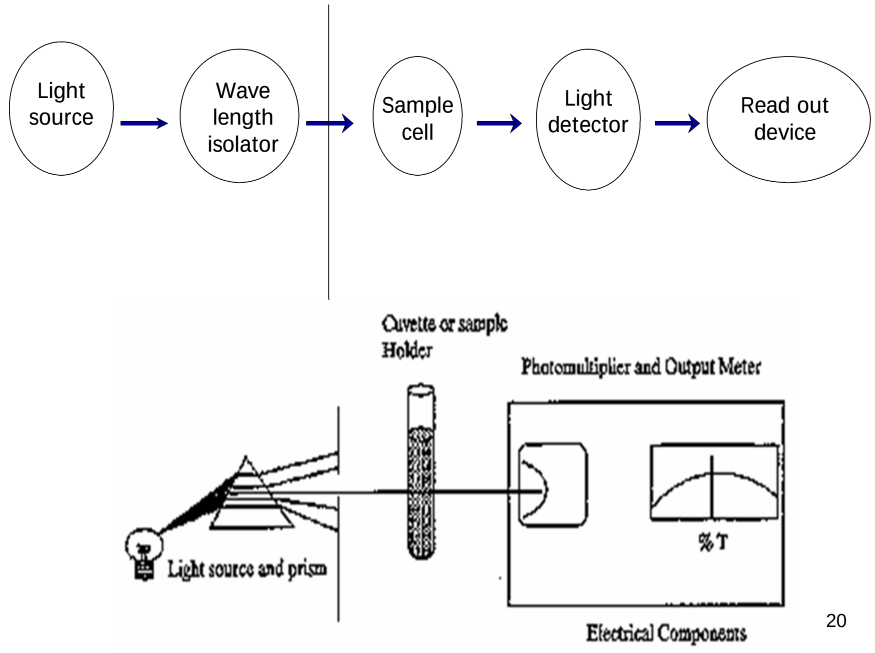
- ***Filter:*** *To produce a monochromatic beam.*
- ***Cuvette:*** *The sample holder, and light should be passed through the transparent side of cuvette, as the opaque side prevents the scattering of light.*
- λ_{max}: *The wavelength which is maximally absorbed by certain substance.*

Procedure:

1) Coloured sample is placed in the cuvette.

2) The sample will absorb some light and transmit the others according to its own colour.

3) The transmitted colour will be detected on the detector by its energy.



Calculations

Beer's Law

When a monochromatic light passes through a colored solution, the amount of light absorbed is directly proportional to the concentration of the colored solute.

$$(I_0 - I_t) \propto C$$

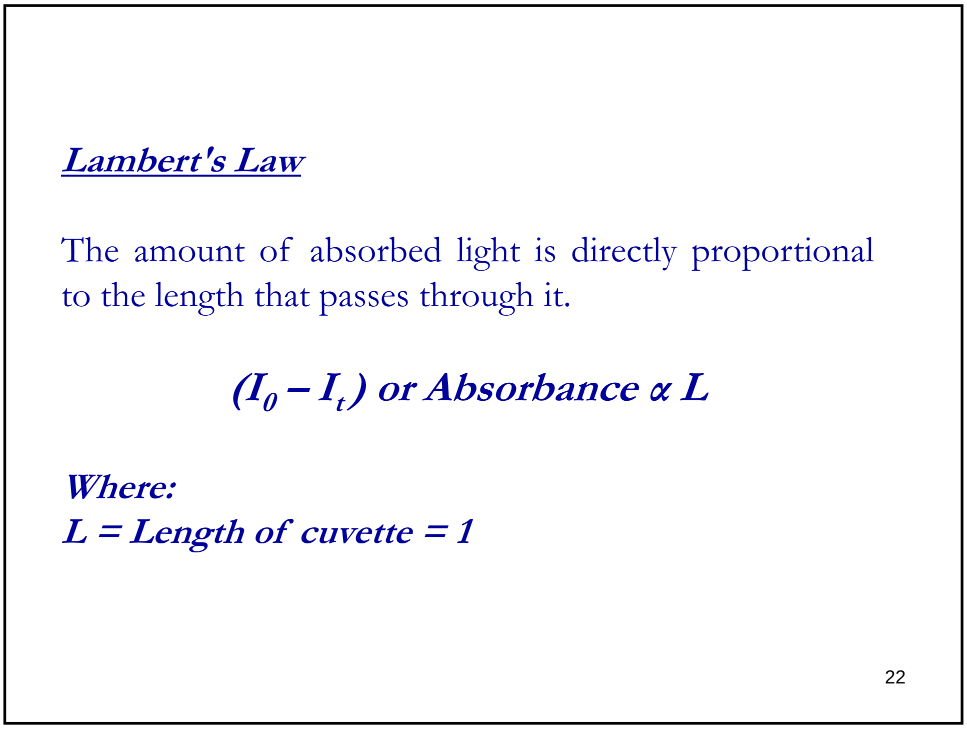
Where:

I_0 = *intensity of light.*

I_t = *intensity of transmitted light.*

$I_0 - I_t$ = *intensity of absorbed light (Absorbance) = A*

C = Concentration.

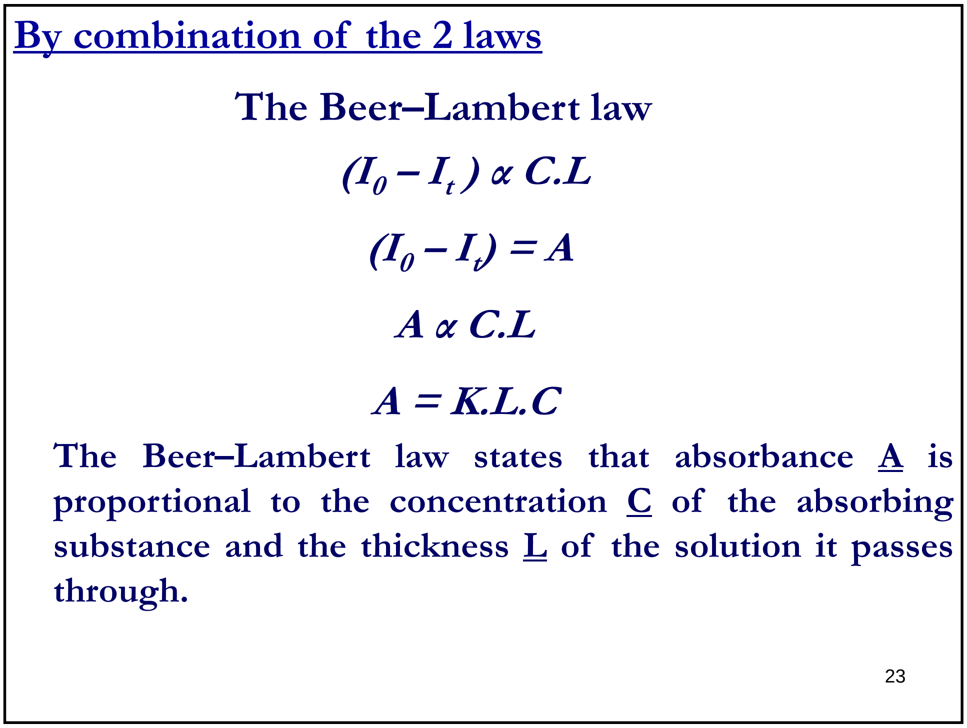


Lambert's Law

The amount of absorbed light is directly proportional to the length that passes through it.

$$(I_0 - I_t) \text{ or Absorbance } \propto L$$

Where:
L = ***Length of cuvette*** $= 1$



By combination of the 2 laws

The Beer–Lambert law

$$(I_0 - I_t) \propto C.L$$

$$(I_0 - I_t) = A$$

$$A \propto C.L$$

$$A = K.L.C$$

The Beer–Lambert law states that absorbance A is proportional to the concentration C of the absorbing substance and the thickness L of the solution it passes through.

$$A = K.L.C$$

A = absorbance.

C = concentration of the absorbing substance.

L = thickness of the solution that light pass through.

K = constant (The absorption coefficient which is dependant on the type of substance and the wavelength used).

L (always) = 1, thus

$$A = K.C$$

We determine the concentration of obtained substance by solution of known concentration of the same substance (standard):

Solution 1 (Standard - Known concentration)

$$A1 = K.C1$$

Solution 2 (Unknown concentration)

$$A2 = K.C2$$

By dividing the two equations:

$$A1/A2 = C1/C2$$

$$A1/A2 = C1/C2$$

A1 =
Absorbance of the standard of the known concentration.
A1 can be known from spectrophotometer.

A2 =
Absorbance of the substance with unknown concentration.
A2 can be known from spectrophotometer.

C1 =
Concentration of standard (known).

C2 =
The unknown concentration which can be obtained using the above equation.

$$C2 = A2 \times C1 / A1$$

$$C2 = A2/A1 \times C1$$

$$C2 = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Conc. of standard}$$

Blank:
For each quantitative assay, blank must be carried out to exclude any colour caused by reagents (Blank contains all reagents except the substance to be measured, which has been replaced by distilled water).

Uses of Spectrophotometeric analysis

- Many analysis methods in medicine and pharmacy utilize this method. Both UV light (λmax 200400nm) and visible light(λmax 400nm------800nm) are used in this method.
- This method is commonly used in the determination of blood chemistry and clinical enzymology
- This method can be used for any molecules which have conjugated double bond (chromogen).

Enzymes play an important role in biochemical analysis. In biological material e. g., in body fluids even tiny quantities of an enzyme can be detected by measuring its catalytic activity. However, enzymes are also used as reagents to determine the concentrations of metabolites e. g., the blood glucose level.

Blood Glucose

Determination of Blood Glucose

Clinical Significance

Normal value:

Fasting blood glucose = 75 - 115 mg %

Increased (Hyperglycaemia):

1) Diabetes mellitus:
   Fasting blood glucose exceeds 200 mg%.
2) pancreatic diseases.
3) Some endocrine disorders such as hyperactivity of the thyroid and pituitary glands.
4) Transient hyperglycaemia often occurs following severe stress as in injury, shock, or severe burns.

Decreased (Hypoglycaemia):

Blood glucose below 40 mg% accompanied by symptoms such as fainting, sweating, hunger, confusion, and violence.

1) Insulin overdose.
2) Severe liver disease.
3) Hypoactivity of the thyroid and pituitary glands.
4) Severe exercises.
5) Glycogen storage diseases.

Principle of analysis

- Two successive reactions. The glucose-specific enzyme glucose oxidase (obtained from fungi) first produces hydrogen peroxide, H_2O_2,which in the second reaction catalyzed by a peroxidase enzyme that oxidizes a colorless precursor into a coloured complex.
- When all of the glucose in the sample has been used up, the amount of dye formed which can be measured on the basis of its light absorption is equivalent to the quantity of glucose originally present. *(i.e. The absorbance of the coloured complex is proportional to the glucose concentration).*

Principle of analysis

Glucose oxidase method

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2.$$

$$H_2O_2 + \text{Oxygen acceptor (e.g. 4-aminoantipyrine)} \xrightarrow{\text{Peroxidase}} \text{Coloured chromagen}.$$

Procedure:

Prepare 3 test tubes labeled test (T), standard (S) and blank (B):

	Test	Standard	Blank
Serum	**10 µl**		
Standard		**10 µl**	
Distilled water			**10 µl**
Working reagent	**1 ml**	**1 ml**	**1 ml**

Mix and incubate for 10 minutes at 25 °C or 5 minutes at 37 °C, and then measure the absorbance of test and standard against reagent blank at 500 nm.

Working reagent contains:

1) Glucose Oxidase.

2) Peroxidase.

3) 4-aminoantipyrine.

Calculation:

Concentration of glucose (mg/dl) =

Absorbance of test/Absorbance of standard
X concentration of standard (mg/dl).

Serum
Total Protein

Determination of Serum Total Protein

Clinical Significance

Normal value:

6.6 – 8.7 g/dl

Increased (Hyperproteinemia):

1) Excessive production: primarily of gamma globulins as in malignancy.

2) Dehydration: as in vomiting, and diarrhea. In this case, the absolute quantity of proteins remains unchanged but the concentration is elevated due to loss of water from the vascular system.

Decreased (Hypoproteinemia):

1) Decreased synthesis as in liver disease.

2) Accelerated catabolism of proteins.

3) Excessive loss as in renal disease (proteins are lost in urine), severe bleeding, and extensive burns.

4) Malnutrition.

The measurement of serum total proteins (albumin and globulin) or plasma total proteins (albumin, globulin and fibrinogen) has only limited clinical value. A fall in albumin concentration may be balanced by a rise in immunoglobulin concentration as in liver diseases.

Principle of analysis

Biuret method:

Alkaline $CuSO_4$ forms a coloured complex with all compounds containing amide bonds. The colour ranges from blue to violet depending on both the concentration and the type of protein determined. The absorbance of the coloured complex is proportional to the total protein concentration.

Procedure:

Prepare 3 test tubes labeled test (T), standard (S) and blank (B):

	Test	Standard	Blank
Serum	**20 µl**		
Standard		**20 µl**	
Distilled water			**20 µl**
Biuret reagent	**1 ml**	**1 ml**	**1 ml**

Mix and incubate for 5 minutes at 25 °C and then measure the absorbance of test and standard against reagent blank at 540 nm.

Biuret reagent contains:

1) Sodium hydroxide.

2) Copper sulphate.

Calculation:

Concentration of total protein (g/dl) =

Absorbance of test/Absorbance of standard X concentration of standard (g/dl).

Serum Albumin

Determination of Serum Albumin

- Albumin is produced entirely by the liver and constitute about 60% of serum total proteins. When the concentration of albumin is significantly reduced, the plasma osmotic pressure is insufficient to draw water from the tissue spaces back into the plasma. This leads to a build-up of fluid in the tissues, referred to as edema.
- Albumin also has an important binding and transport function. It binds and transports substances including calcium, bilirubin, fatty acids, urate, hormones and also drugs such as penicillin, sulphonamides and barbiturates. When albumin levels are reduced, toxic effects can develop from an increase in unbound substances.

Clinical Significance

Normal value:

3.8 – 5.4 g/dl

Increased:

1) Serum albumin are rarely increased except artifactually by prolonged venous stasis.

Decreased:

1) Decreased synthesis as in liver disease.
2) Increased loss through urine as in kidney diseases.
3) Increased body need for the protein as in infections and malignant diseases.
4) Low protein intake.

Principle of analysis

Bromocresol green (BCG) method:

Serum albumin binds selectively to bromocresol green at a slightly acidic pH (4.2), and produces a colour change of the indicator from yellowish-green to greenish blue. The absorbance of the coloured complex is proportional to the albumin concentration.

Procedure:

Prepare 3 test tubes labeled test (T), standard (S) and blank (B):

	Test	Standard	Blank
Serum	**10 µl**		
Standard		**10 µl**	
Distilled water			**10 µl**
Reagent	**1 ml**	**1 ml**	**1 ml**

Mix and incubate for 10 minutes at 25 °C and then measure the absorbance of test and standard against reagent blank at 630 nm.

Reagent contains:

1) Bromocresol green (BCG) in citrate buffer (pH = 4.2).

Calculation:

Concentration of albumin (g/dl) =

Absorbance of test/Absorbance of standard

X concentration of standard (g/dl).

Calculation of serum globulin

Serum globulin =

Serum total protein – Serum albumin

Serum Urea

Determination of Serum Urea

- Urea is the main end product of protein metabolism in the body. Determination of serum urea and serum creatinine are the two most commonly ordered tests to detect the kidney's ability to excrete metabolic waste. Increase in these values are used as indicators for the need for dialysis in chronic kidney failure patients.

- Urea is only synthesized in the liver and is eliminated through the kidney. Blood urea determination is considered as a link between liver and kidney.

Clinical Significance

Normal value:

Serum urea = 15 - 45 mg %

Increased:

1) Damaged renal functions: nephritis, acute renal failure and urinary tract obstruction.
2) Diabetes mellitus, due to deficiency of insulin (an anabolic hormone) and increased destruction of protein for energy demand.
3) Any condition associated with increased protein breakdown such as severe infections and surgical operations.
4) High doses of highly catabolic drugs e.g. cortisone.

Decreased:

1) Malnutrition.
2) Severe liver disease.

Principle of analysis

Berthelot method

- Urea is cleaved by the action, of urease enzyme giving ammonia.
- Ammonia by addition of hypochlorite gives chloramine which in the presence of excess phenol gives indophenol (blue-green colour). The absorbance of the coloured complex is proportional to the urea concentration.

Procedure:

Prepare 3 test tubes labeled test (T), standard (S) and blank (B):

	Test	Standard	Blank
Serum	**10 µl**		
Standard		**10 µl**	
Distilled water			**10 µl**
Reagent 1	**1 ml**	**1 ml**	**1 ml**

Mix and incubate for 5 minutes at 25 °C.

	Test	Standard	Blank
Reagent 2	**1 ml**	**1 ml**	**1 ml**

Mix and incubate for 10 minutes at 25 °C and then measure the absorbance of test and standard against reagent blank at 600 nm.

Reagent 1 contains:

1) Urease enzyme.

Reagent 2 contains:

1) Sodium hypochlorite.

2) Phenols.

Calculation:

Concentration of urea (mg/dl) =

Absorbance of test/Absorbance of standard X concentration of standard (mg/dl).

Serum Uric Acid

Determination of Serum Uric Acid

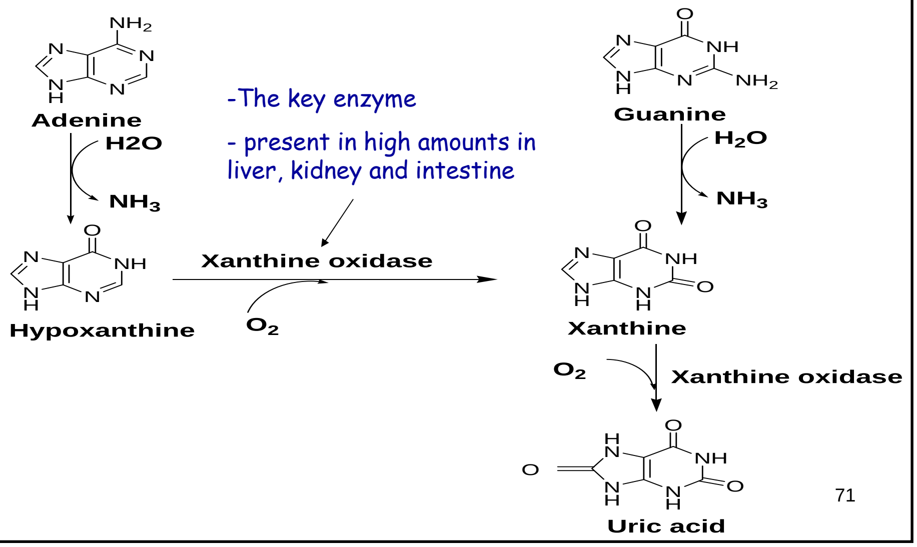
- Uric acid is the metabolic end product of purine catabolism. Uric acid can be formed from purine-containing compounds present in food.
- Richest are organ meats such as liver, heart, kidneys, next come poultry and fish. There are only small amounts in vegetables and cereals, little or none in fruits and milk products.
- Mammals, except humans, have uricase enzyme which converts uric acid to soluble allantoin.

De novo synthesis of uric acid

$$\text{Glucose} \xrightarrow{\text{HMP Shunt}} \text{Ribose-5-P} \xrightarrow[\text{PPRP synthetase}]{\text{Purine synthesis}} \text{Purine} \xrightarrow{\text{Catabolism}} \text{Uric acid}$$

N.B. PPRP= Pyrophosphate ribose phosphate

Biosynthesis of uric acid



-The key enzyme

- present in high amounts in liver, kidney and intestine

Clinical Significance

Normal value:

Serum Uric Acid

Males: 3.5 - 7 mg %

Females: 2.5 – 6 mg%

Increased:

Hyperuricemia (Gout):

- It is the increase of blood uric acid level above 7.0 mg%.
- It is a clinical condition characterized by hyperuricemia with precipitation of deposits of uric acid needle crystals in joints and cartilage with acute recurrent attacks of arthritis. These urate deposition is called tophi. Since this is an inflammatory condition, cells undergo anaerobic metabolism with production of lactic acid, this makes the medium more acidic that favours precipitation of more uric acid crystals and so gout aggravates.

In joints:
Urate crystals will be phagocytosed by leukocytes leads to acute inflammatory reactions called acute gouty arthritis. The joints that firstly affected are the small joints especially those of big toes

In kidneys:
Deposition of uric acid tophi may leads to uric acid stone.

In cartilage:
as in the ear leads to destructions of cartilage.

Decreased:

Hypouricemia:

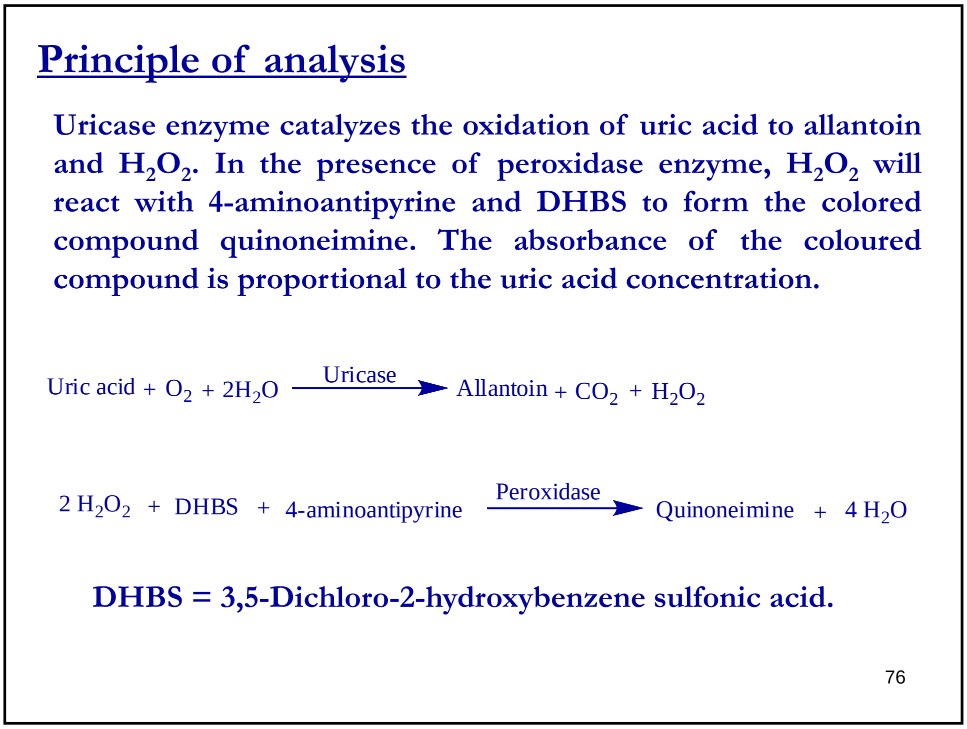
It is the decrease of blood uric acid level below 2 mg%.

Causes:

1- Deficiency of xanthine oxidase enzyme which leads to increase in xanthine and hypoxanthine excretion.

2- Drugs: such as acetylsalicylic acid (Asprin).

High dose of Aspirin actually blocks reabsorption of uric acid by the kidneys, causing uric acid to be excreted in the urine and lowering the blood level of uric acid.



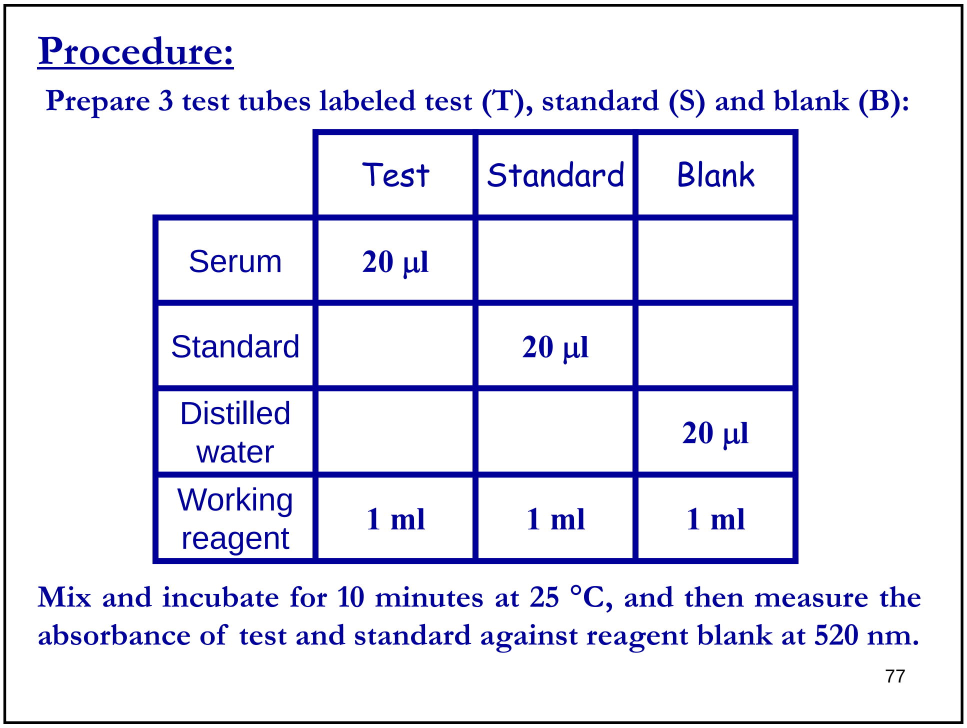
Principle of analysis

Uricase enzyme catalyzes the oxidation of uric acid to allantoin and H_2O_2. In the presence of peroxidase enzyme, H_2O_2 will react with 4-aminoantipyrine and DHBS to form the colored compound quinoneimine. The absorbance of the coloured compound is proportional to the uric acid concentration.

$$\text{Uric acid} + O_2 + 2H_2O \xrightarrow{\text{Uricase}} \text{Allantoin} + CO_2 + H_2O_2$$

$$2\,H_2O_2 + \text{DHBS} + \text{4-aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4\,H_2O$$

DHBS = 3,5-Dichloro-2-hydroxybenzene sulfonic acid.



Procedure:

Prepare 3 test tubes labeled test (T), standard (S) and blank (B):

	Test	Standard	Blank
Serum	**20 µl**		
Standard		**20 µl**	
Distilled water			**20 µl**
Working reagent	**1 ml**	**1 ml**	**1 ml**

Mix and incubate for 10 minutes at 25 °C, and then measure the absorbance of test and standard against reagent blank at 520 nm.

Working reagent contains:

1) *Uricase.*
2) *Peroxidase*
3) *4-aminoantipyrine.*
4) *DHBS.*

Calculation:

Concentration of glucose (mg/dl) =

Absorbance of test/Absorbance of standard
X concentration of standard (mg/dl).

Serum Creatinine

Serum Creatinine

&

Creatinine Clearance Test

- **The NPN (Non Protein Nitrogenous compounds) includes urea, uric acid, creatinine, creatine,, amino acids, ammonia. Creatinine is the least variable of the non protein nitrogenous (NPN) substances in the serum and is a sensitive indicator of renal function.**

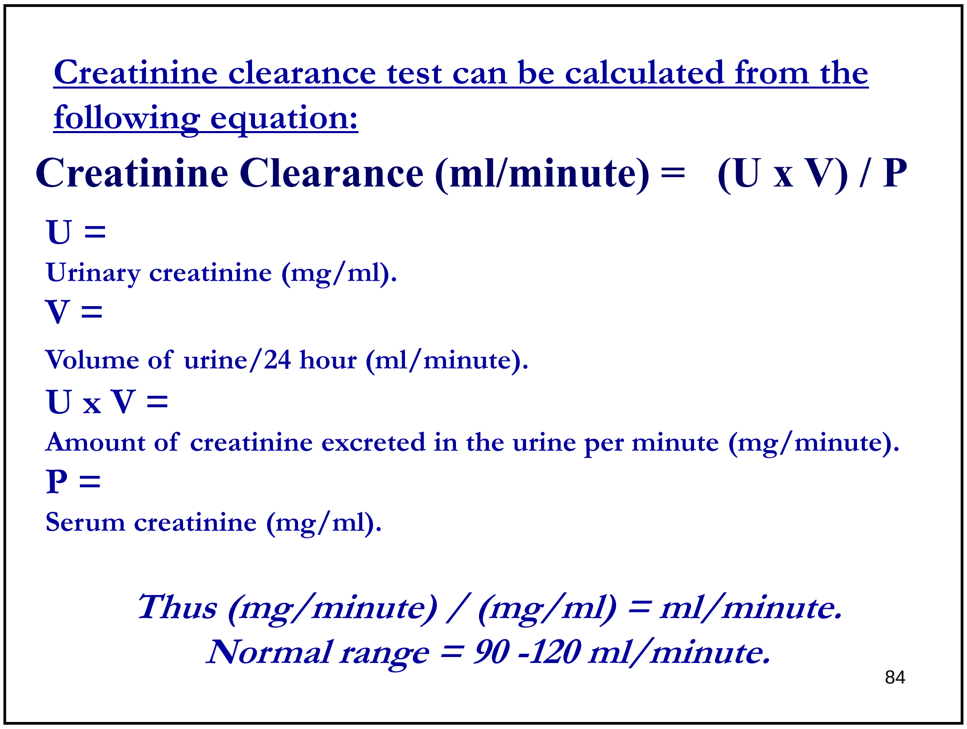
- **Creatinine is the end product of muscle metabolism that is liberated from the muscle and excreted in the urine at a virtually constant rate.**

- Creatine is the only precursor of creatinine. Creatine is a methylguanido acetic acid and stored in energy reserve form (creatine phosphate) in muscle. Creatinine (creatine anhydride) is formed in muscles from creatine phosphate by irreversible, non enzymatic dehydration.

- Creatinine is correlated with changes in the serum urea for diagnostic purposes but is more accurate as an index of glomerular filtration rate (GFR) because it is produced in amounts related to muscle mass, which changes very little while the serum urea level changes in response to many factors, such as high/low protein diet, or dialysis. Thus level of creatinine will be used instead as a true measure of the renal damage.

- Creatinine is filtered through glomerulus of the kidney, and it is not reabsorbed by the tubules. Thus the rate of excretion in the urine is also constant from day to day and proportionate to muscle mass. Serum creatinine levels depends on the balance between their production and excretion.

- Clearance test measure the amount of blood which could theoretically be completely cleared of a creatinine per minute. Creatinine clearance test is a convenient method of obtaining a fairly accurate estimation of the glomerular filtration rate (GFR). This test has particular value in the general assessment of renal function specially in cases when plasma analysis are invalid (eg. After dialysis), or when the serum urea has been lowered by a low protein diet.

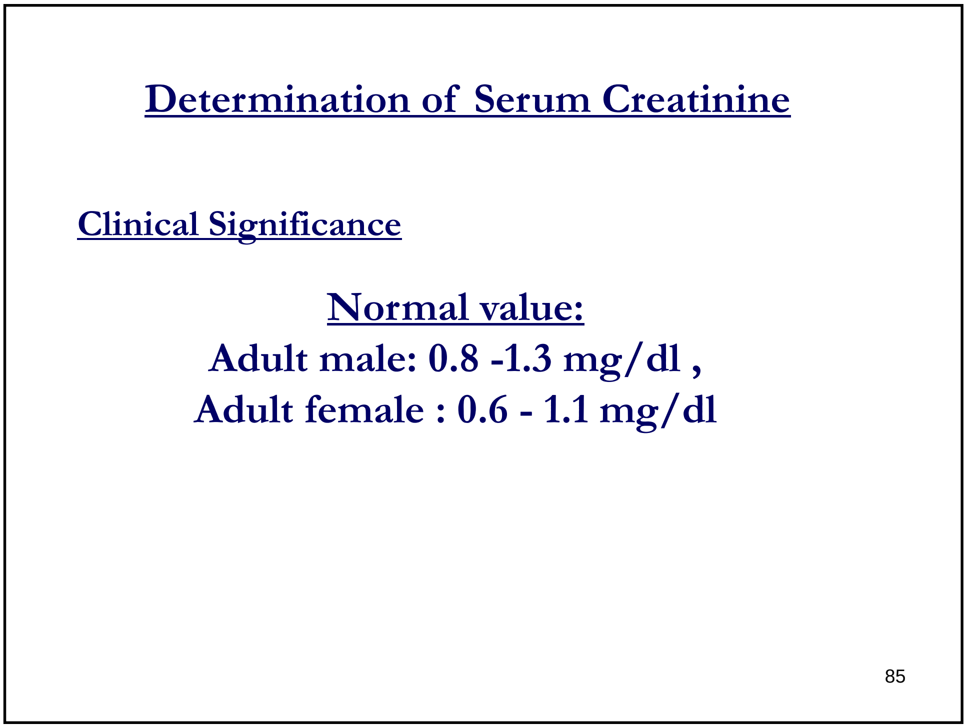


Creatinine clearance test can be calculated from the following equation:

Creatinine Clearance (ml/minute) = (U x V) / P

U =
Urinary creatinine (mg/ml).
V =
Volume of urine/24 hour (ml/minute).
U x V =
Amount of creatinine excreted in the urine per minute (mg/minute).
P =
Serum creatinine (mg/ml).

Thus (mg/minute) / (mg/ml) = ml/minute.
Normal range = 90 -120 ml/minute.



Determination of Serum Creatinine

Clinical Significance

Normal value:
Adult male: 0.8 -1.3 mg/dl ,
Adult female : 0.6 - 1.1 mg/dl

Increased:

Serum creatinine is elevated due to any impairment of the glomeruli resulting in retention of creatinine and increasing its concentration in serum. Thus serum creatinine is increasing in acute or chronic renal insufficiency, urinary tract obstruction and impairment of renal function induced by certain drugs.

1) Serum creatinine is also elevated by increasing muscle wasting as in case of hyperthyroidism, diabetes mellitus and starvation.

Decreased:

Serum creatinine is decreased in muscle dystrophy.

Principle of analysis

Jaffe's reaction is the most sensitive reaction used to determine creatinine.

Creatinine + alkaline solution of picrate → Creatinine picrate complex (reddish-orange)

(Orange complex measured calorimetrically at 490 nm)

The absorbance of the coloured complex is proportional to the creatinine concentration.

Procedure:

Prepare 3 test tubes labeled test (T), standard (S) and blank (B):

	Test	Standard	Blank
Serum	**100 µl**		
Standard		**100 µl**	
Distilled water			**100 µl**
Working reagent	**1 ml**	**1 ml**	**1 ml**

Mix and incubate for 15 minutes at 25 °C, and then measure the absorbance of test and standard against reagent blank at 540 nm.

Working reagent contains:

1) Picric acid.

2) Sodium hydroxide.

Calculation:

Concentration of creatinine (mg/dl) =

Absorbance of test/Absorbance of standard

X concentration of standard (mg/dl).

Serum Calcium

MINERALS

Minerals could be divided, according to the body requirements, into two groups:

I- Macrominerals:

They are required in amounts greater than 100 mg/day.

They include 6 elements:

Calcium, Phosphorous, Magnesium, Sodium, Potassium, and Chloride.

II- Microminerals (Trace elements)

They are required in amounts less than 100 mg/day.

They include 10 elements:

Iron, Copper, Zinc, Iodine, Selenium, Manganese, Cobalt, Chromium, Fluoride and Lithium.

Calcium

Sources:

1- Milk and milk products (the richest source).

2- Beans, egg yolk and leafy vegetables.

Requirements:

Adults require about 800 mg/day while children, pregnant and lactating women require about 800-1200 mg/day.

Absorption

Calcium is absorbed by active transport mechanism in small intestine in presence of calcium binding protein.

Factors affecting absorption of calcium

a) Factors increase calcium absorption:

1- High protein diet: ***amino acids form with calcium a soluble calcium salt that is easily absorbed.***

2- High dietary lactate or citrate: ***that form soluble salts with calcium.***

b) Factors inhibit calcium absorption:

1- High dietary phosphates and oxalate: ***form insoluble salts with calcium.***

2- Impaired fat absorption: ***fatty acids form insoluble calcium salts.***

Distribution

- **Calcium is present in the body in large amounts than any other element (about 1200 gm).**
- **Most of calcium (about 99%) is present in the skeleton (bones and teeth). The remaining 1% is present in body fluids and other tissues.**

Plasma calcium

Blood calcium presents entirely in the plasma (Red blood cells does not contain calcium).

Plasma calcium is present in three forms:

1- Ionized calcium [diffusible] (50%):

- ***It is the physiological active fraction.***
- ***Its deficiency causes tetany.***

2- Non-ionized [diffusible] (5%):

- ***Complexes with organic acids e.g. citrate.***

3- Non-ionized [non-diffusible] (45%):

- ***It is bound with albumin and its deficiency causes no tetany.***

Functions of calcium

1- Non-ionized calcium enters in the structure of bones and teeth.

2- Ionized calcium is important for:

- Transmission of nerve impulses.
- Decrease of neuromuscular excitability.
- Blood clotting.
- Milk clotting in infant stomach.
- Activation of certain enzymes e.g. lipase enzyme.

Relation between parathyroid hormone, vitamin D and calcium

- **Parathyroid hormone (PTH) activate the conversion of vitamin D into 1,25-dihydroxycholecalciferol (calcitriol) in the kidney.**
- **Calcitriol stimulates synthesis of calcium binding protein.**

Hormonal regulation of plasma calcium level

Parathyroid hormone and calcitriol (active vitamin D) increase plasma calcium level through:

- ***Increase mobilization of calcium from bones (bone resorption).***
- ***Increase absorption of calcium from intestine.***
- ***Increase reabsorption of calcium by renal tubules.***

Calcitonin and Katacalcin (hormones secreted from parafollicular cells or C-cells of thyroid glands in response to hypercalcemia) decrease plasma calcium level through:

- ***Inhibition of its mobilization from bones.***
- ***Increasing calcium deposition in bones.***

Osteoporosis

- ***It is caused by increased activity of osteoclasts (bone eating cells) and reduced activity of osteoblasts (bone forming cells).***
- ***Estrogen hormone inhibits osteoclasts. Thus after menopause, osteoporosis is common due to deficiency of estrogen.***

Determination of Serum Calcium

Clinical Significance

Normal value:

9 - 11 mg/dl

Principle of analysis

Calcium ions form a violet complex with O-Cresolphthalein complexone in alkaline medium.

The absorbance of the coloured complex is proportional to the calcium concentration.

Procedure:

Prepare 3 test tubes labeled test (T), standard (S) and blank (B):

	Test	Standard	Blank
Serum	**10 µl**		
Standard		**10 µl**	
Distilled water			**10 µl**
Working reagent	**0.5 ml**	**0.5 ml**	**0.5 ml**

Mix and measure the absorbance of test and standard against reagent blank at 570 nm.

Working reagent contains:

1) O-Cresolphthalein complexone in buffer (pH 11.1).

Calculation:

Concentration of calcium (mg/dl) =

Absorbance of test/Absorbance of standard
X concentration of standard (mg/dl).

Serum Magnesium

Macrominerals

Magnesium

Sources:

Leafy green vegetables.

Distribution:

- Mostly 70% of magnesium present in skeleton (bone and teeth).

- The remaining 30% is present in body fluids and other tissues mostly intracellular.

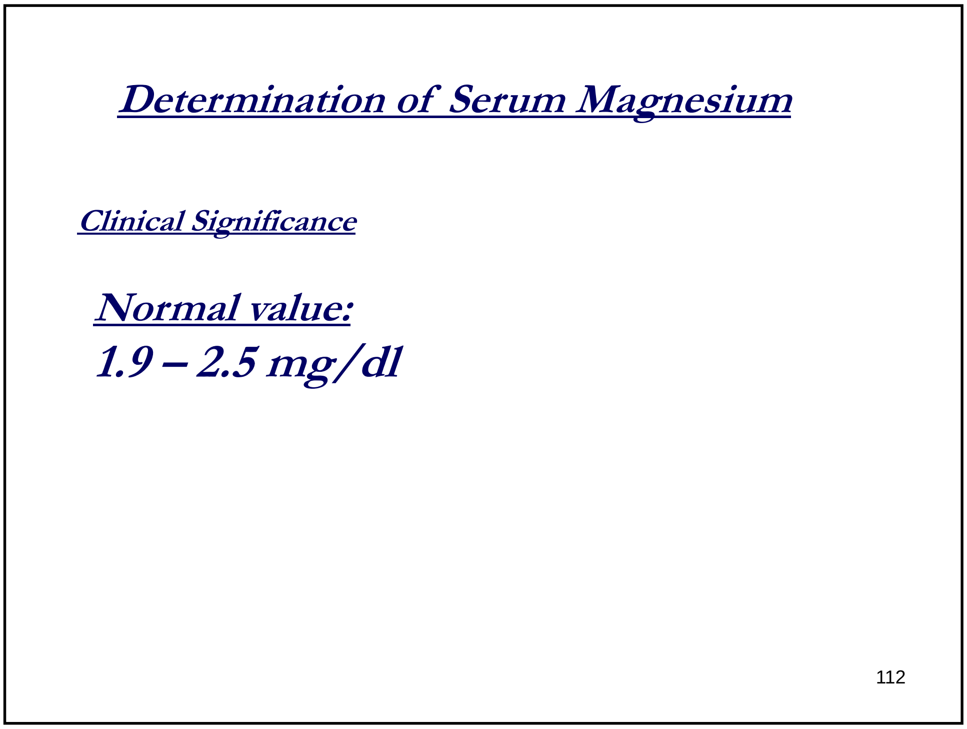
Functions of magnesium:

1- It enters in the structure of skeleton (bones and teeth).

2- It activates many enzymes such as kinases enzymes.

3- It is required for the active transport of other cations (Ca^{2+}, Na^{+} and K^{+}) across cell membranes.

4- It is important for muscle contraction, nerve impulse transmission and it decreases neuromuscular excitability.

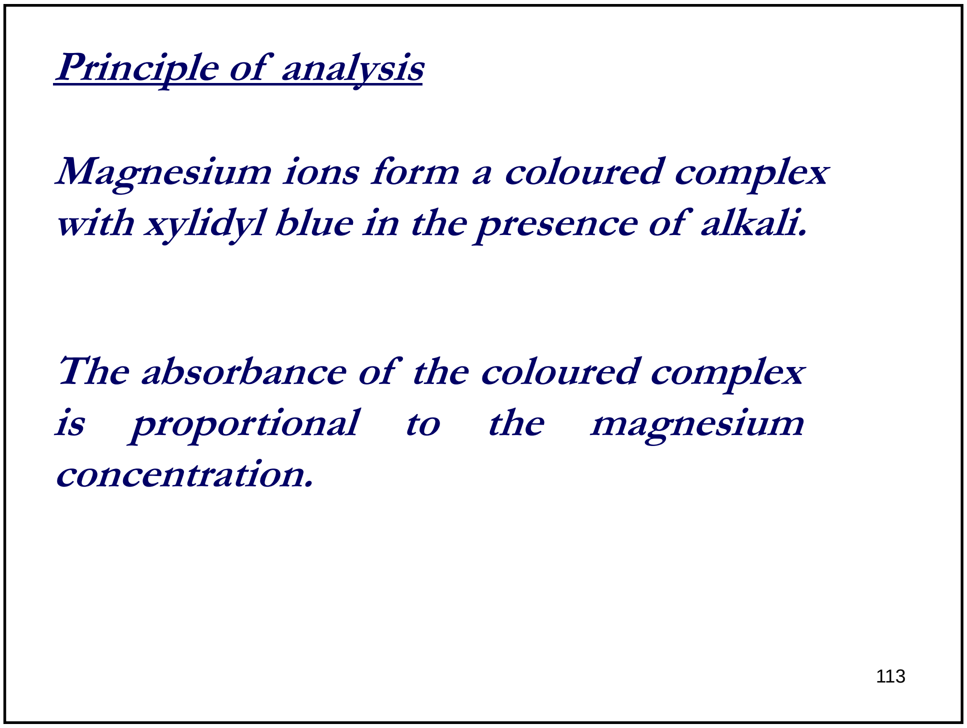


Determination of Serum Magnesium

Clinical Significance

Normal value:

1.9 – 2.5 mg/dl



Principle of analysis

Magnesium ions form a coloured complex with xylidyl blue in the presence of alkali.

The absorbance of the coloured complex is proportional to the magnesium concentration.

Procedure:

Prepare 3 test tubes labeled test (T), standard (S) and blank (B):

	Test	Standard	Blank
Serum	**10 µl**		
Standard		**10 µl**	
Distilled water			**10 µl**
Working reagent	**1 ml**	**1 ml**	**1 ml**

Mix and incubate for 10 minutes at 25 °C and measure the absorbance of test and standard against reagent blank at 546 nm.

Working reagent contains:

1) Xylidyl blue in Tris buffer (pH 11).

Calculation:

Concentration of magnesium (mg/dl) =

Absorbance of test/Absorbance of standard
X concentration of standard (mg/dl).

Serum Triglycerides

Determination of Serum Triglycerides

- Plasma lipids include triglycerides, cholesterol, phospholipids and free fatty acids.
- Lipids are immiscible with water. Thus, they must combine with proteins to form lipoproteins.

Triglycerides are tranported in blood by the following two lipoprotein fractions:

1- Chylomicrons:

Transport exogenous (dietary) triglycerides.

2- VLDL:

Transport endogenous triglycerides from liver to peripheral tissues.

Clinical Significance

Normal value:

Serum triglycerides
= 35 - 165 mg %

- Triglycerides are fats that provide energy for the cell. Like cholesterol, they are delivered to the body's cells by lipoproteins in the blood.

- The increases in serum triglycerides are relatively non-specific.

For example:

1) **A diet with a lot of saturated fats or carbohydrates will raise the triglyceride levels.**
2) **Liver dysfunction resulting from hepatitis, extra hepatic biliary obstruction or cirrhosis will also raise the triglyceride levels.**
3) **Diabetes mellitus is associated with triglycerides increase as well.**

Clinical diagnosis should not be made on a single test result; it should integrate clinical and other laboratory data.

Principle of analysis

Sample triglycerides incubated with lipoprotein lipase (LPL), liberate glycerol and free fatty acids. Glycerol is converted to glycerol-3-phosphate (G3P) by glycerol kinase and ATP. Glycerol-3-phosphate (G3P) is then converted by glycerol-3-phosphate oxidase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2).

In the last reaction, hydrogen peroxide (H_2O_2) reacts with 4-aminophenazone and p-chlorophenol in presence of peroxidase (POD) to give a red coloured compound. The absorbance of the coloured compound is proportional to the triglycerides concentration.

$$\text{Triglycerides} + H_2O \xrightarrow{LPL} \text{Glycerol} + \text{Free fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{G3P} + \text{ADP}$$

$$\text{G3P} + O_2 \xrightarrow{GPO} \text{DAP} + H_2O_2$$

$$H_2O_2 + \text{4-aminophenazone} + \text{p-Chlorophenol} \xrightarrow{POD}$$

Quinoneimine
(coloured compound)

Procedure:

Prepare 3 test tubes labeled test (T), standard (S) and blank (B):

	Test	Standard	Blank
Serum	10 µl		
Standard		10 µl	
Distilled water			10 µl
Working reagent	1 ml	1 ml	1 ml

Mix and incubate for 10 minutes at 25 °C, and then measure the absorbance of test and standard against reagent blank at 540 nm.

Working reagent contains:

1) *Lipases.*
2) *Glycerol kinase.*
3) *GPO.*
4) *POD.*
5) *ATP.*
6) *P-chlorophenol.*
7) *4-aminoantipyrine.*

Calculation:

Concentration of triglycerides (mg/dl) =

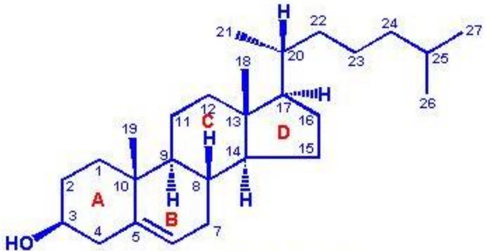
Absorbance of test/Absorbance of standard X concentration of standard (mg/dl).

Serum
Total Cholesterol

Determination of Serum Total Cholesterol

- Cholesterol is an extremely important biological molecule that has roles in membrane structure as well as being a precursor for the synthesis of the steroid hormones and bile acids.
- Both dietary cholesterol and that synthesized *de novo* are transported through the circulation in lipoprotein particles. The same is true of cholesterol esters, the form in which cholesterol is stored in cells.

The synthesis and utilization of cholesterol must be tightly regulated in order to prevent over-accumulation and abnormal deposition within the body. Of particular importance is the abnormal deposition of cholesterol and cholesterol-rich lipoproteins in the coronary arteries. Such deposition, eventually leading to atherosclerosis, is the leading contributory factor in heart diseases.



Cholesterol

Cholesterol is tranported in blood by the following two lipoprotein fractions:

1- LDL:

Transports cholesterol from liver to peripheral tissues.

2- HDL:

Reverse cholesterol transport. It transports cholesterol from tissues to liver (cholesterol scavenger).

Clinical Significance

Normal value:
Serum total cholesterol
< 200 mg %

Plasma lipoproteins and atherosclerosis

- Atherosclerosis is a chronic disease in which deposits of cholesterol, and cholesterol esters accumulate in the inner surfaces of large and medium sized arteries. As the disease progress, the deposits reduce or even stop the flow of blood causing *coronary artery disease*.
- The heart cells supplied with blood by affected artery will be deprived of oxygen and nutrients and rapidly die. This is called *myocardial infarction*, where part of heart muscle becomes nonfunctional.

- Since the LDL represents the transport of cholesterol to the tissues and HDL represents the removal of cholesterol from tissues thus:

1) **Decreased HDL is associated with atherosclerosis and coronary heart disease.**
2) **Increased HDL represents protection against atherosclerosis and coronary heart disease.**
3) **LDL/HDL ratio helps in predicting atherosclerosis and coronary heart disease:**

↑ LDL/HDL → *Atherosclerosis*
↓ LDL/HDL → *No atherosclerosis*

Estrogens and exercises cause increasing of HDL, thus premenopausal women and persons doing exercises appear to be protected from coronary heart diseases.

Also, diet rich in polyunsaturated fatty acids plays an important role in increasing HDL.

Principle of analysis

Free and esterified cholesterol form hydrogen peroxide (H_2O_2) by the action of cholesterol esterase and cholesterol oxidase.

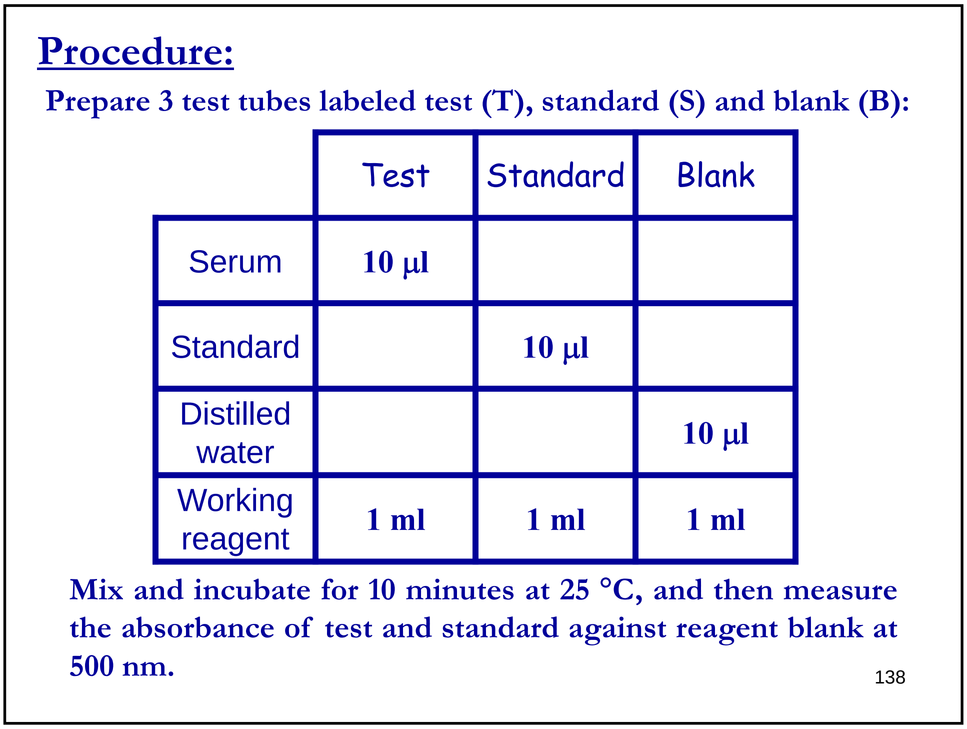
H_2O_2 reacts with 4-aminoantipyrine and phenol to form quinoneimine by the aid of peroxidase enzyme (POD).

$$\text{Cholesterol ester} + H_2O \xrightarrow{\textit{Cholesterol esterase}} \text{Cholesterol} + \text{Free fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\textit{Cholesterol oxidase}} \text{Cholesterol-3-one} + H_2O_2$$

$$H_2O_2 + \text{4-aminophenazone} + \text{Phenol} \xrightarrow{\textit{POD}}$$

**Quinoneimine
(coloured compound)**

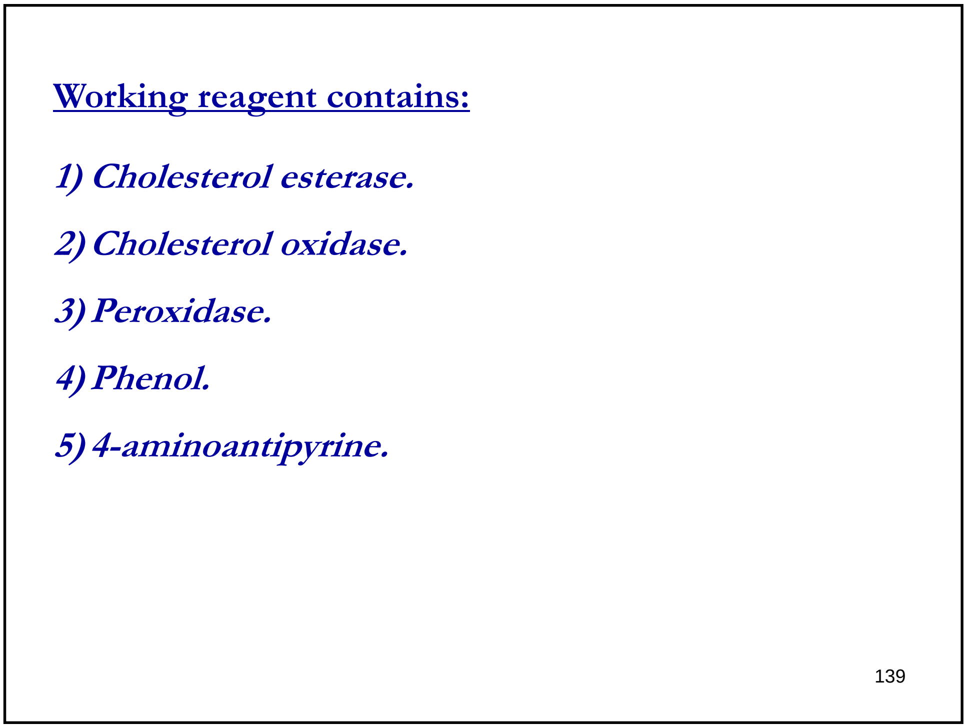


Procedure:

Prepare 3 test tubes labeled test (T), standard (S) and blank (B):

	Test	Standard	Blank
Serum	**10 µl**		
Standard		**10 µl**	
Distilled water			**10 µl**
Working reagent	**1 ml**	**1 ml**	**1 ml**

Mix and incubate for 10 minutes at 25 °C, and then measure the absorbance of test and standard against reagent blank at 500 nm.



Working reagent contains:

1) Cholesterol esterase.

2) Cholesterol oxidase.

3) Peroxidase.

4) Phenol.

5) 4-aminoantipyrine.

Calculation:

Concentration of total cholesterol (mg/dl) =

Absorbance of test/Absorbance of standard

X concentration of standard (mg/dl).

Serum

HDL Cholesterol & Calculation of LDL Cholesterol

Determination of
Serum HDL-Cholesterol &
Calculation of LDL-Cholesterol

Clinical Significance

Normal value:
Serum HDL - cholesterol
> 60 mg %

Since HDL-cholesterol level is inversely related to the tendency of heart disease, its estimation is useful for diagnosis of atherosclerosis.

Principle of analysis

The VLDL, LDL- cholesterol and Chylomicrons fractions are removed by precipitation with phosphotungestate and magnesium ions. After centrifugation, the supernatant is used for estimation of HDL-cholesterol in the same manner as Total Cholesterol.

Procedure:

I- Precipitation

1- Pipette 200 µl of serum sample into centrifuge tube.

2- Add 0.5 ml of precipitation reagent.

3- Mix and allow to stand for 10 minutes.

4- Centrifuge for 10 minutes at 4000 rpm.

5- Take the supernatant to determine HDL-cholesterol.

Precipitation reagent contains phosphotungestic acid and magnesium ions.

II- Assay *(exactly as cholesterol determination)*

Prepare 3 test tubes labeled test (T), standard (S) and blank (B):

	Test	Standard	Blank
Supernatant	**10 µl**		
Standard		**10 µl**	
Distilled water			**10 µl**
Working reagent	**1 ml**	**1 ml**	**1 ml**

Mix and incubate for 10 minutes at 25 °C, and then measure the absorbance of test and standard against reagent blank at 500 nm.

Calculation:

Concentration of HDL-cholesterol (mg/dl) =

Absorbance of test/Absorbance of standard

X concentration of standard (mg/dl).

Calculation of LDL -Cholesterol

Friedewald Method

The LDL-cholesterol can be calculated using Friedewald equation:

LDL-cholesterol (mg/dl) =

Total cholesterol – HDL-cholesterol – Triglycerides/5

Triglycerides/5 is used as an estimate of VLDL-cholesterol concentration.

It assumes,

First, that virtually all of the plasma TG is carried on VLDL,

Second, that the TG : cholesterol ratio of VLDL is constant at about 5:1 (Friedewald et al. 1972).

Electrophoresis

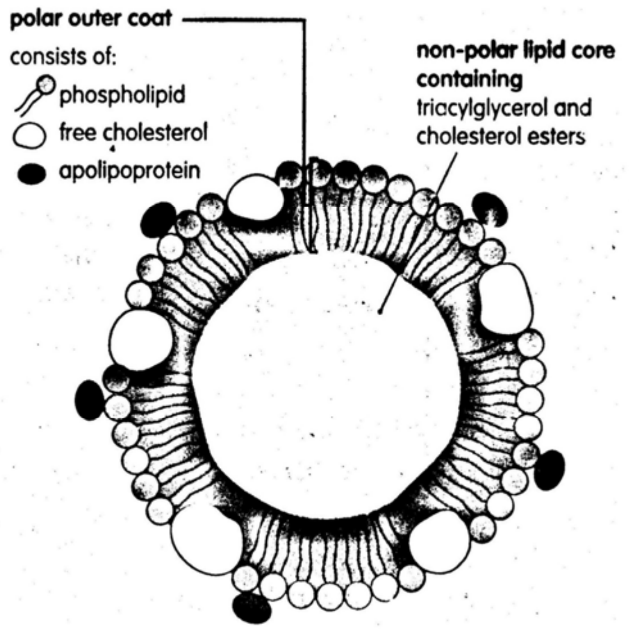
- *Electrophoresis is the movement of ampholytes (amino acids, protein and nucleic acids) in the electric field on the basis of charge or on the basis of size.*
- *Ampholytes: the substances which have pH dependent charges.*

1-Lipoprotein electrophoresis

- Fat absorbed from intestine (dietary lipids) and lipids synthesized in the liver and adipose tissues must be transported between various tissues and organs for utilization and storage.

- Plasma lipids include triglycerides, cholesterol, phospholipids and free fatty acids.

- Lipids are immiscible with water. Thus, they must combine with proteins to form lipoproteins.
- Lipoproteins are a combination of lipid and protein. Lipoproteins consist of a lipid core of mainly non polar triglycerides and cholesterol surrounded by a surface layer of more polar phospholipids and protein fraction known as apoprotein or apolipoprotein.



- *Lipoproteins are classified into groups in order of decreasing size and increasing density.*

- *Apoproteins not only serve for solubility purposes, but rather function as recognition molecules for the membrane receptors and enzymes that are involved in lipid exchange.*

Functions of lipoproteins

Each fraction of the plasma lipoproteins contains almost all types of lipids i.e. triglycerides, phospholipids, cholesterol, and free fatty acids.

They differ in:

1- The main lipid content of each fraction.

2- Source of each fraction.

3- Types & amounts of associated proteins (apolipoproteins).

Fraction	Source	Main Lipid	Apolipoproteins	
			Amounts	Types
Chylomicrons	Intestine	TG	2%	A, B_{48}, C and E.
VLDL	Liver	TG	10%	B_{100}, C and E.
LDL	Blood from chylomicrons and VLDL	Cholesterol, Cholesteryl esters Phospholipids	22%	B_{100}
HDL	Liver	Cholesterol, Cholesteryl esters Phospholipids	50%	A, C, D and E
FFA-Albumin	Adipose tissue	FFA	99%	Albumin

Briefly

Chylomicrons:

Transport exogenous (dietary) triglycerides.

VLDL:

Transport endogenous triglycerides from liver to peripheral tissues.

LDL:

Transport cholesterol from liver to peripheral tissues.

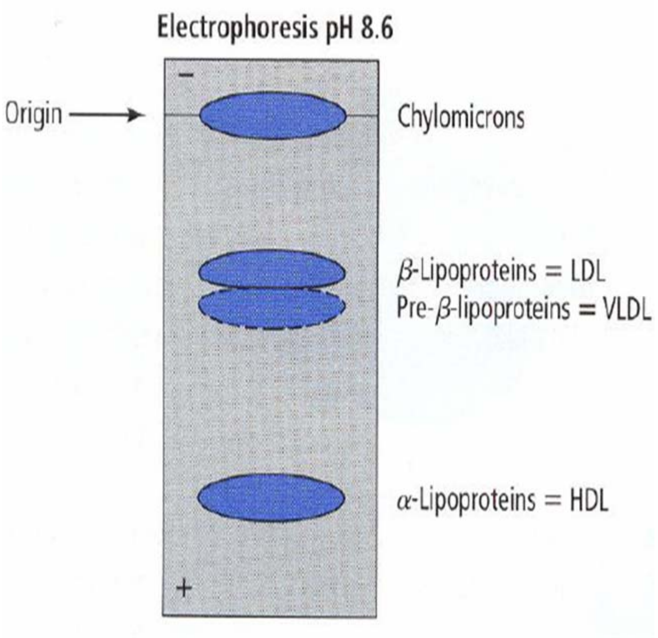
HDL:

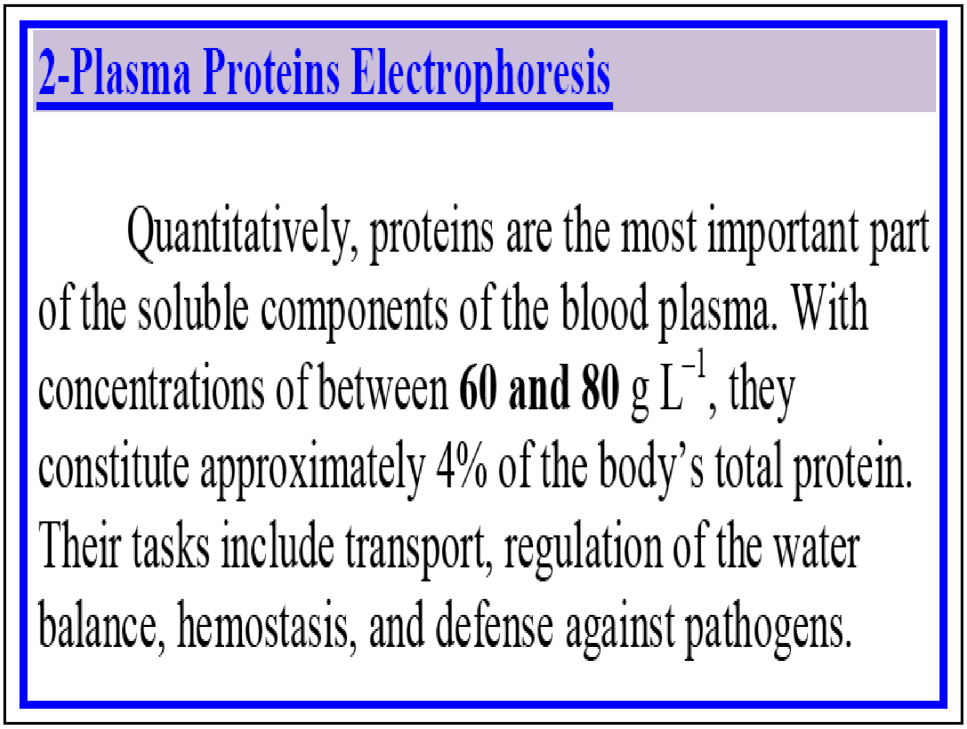
Reverse cholesterol transport. It transport cholesterol from tissues to liver (cholesterol scavenger).

Determination of lipoproteins by the chemical means can not differentiate between different types of hyperlipoproteinemia.

So, determination of plasma lipoprotein by electrophoresis is preferred because it can differentiate between the different types and thus help the treatment.

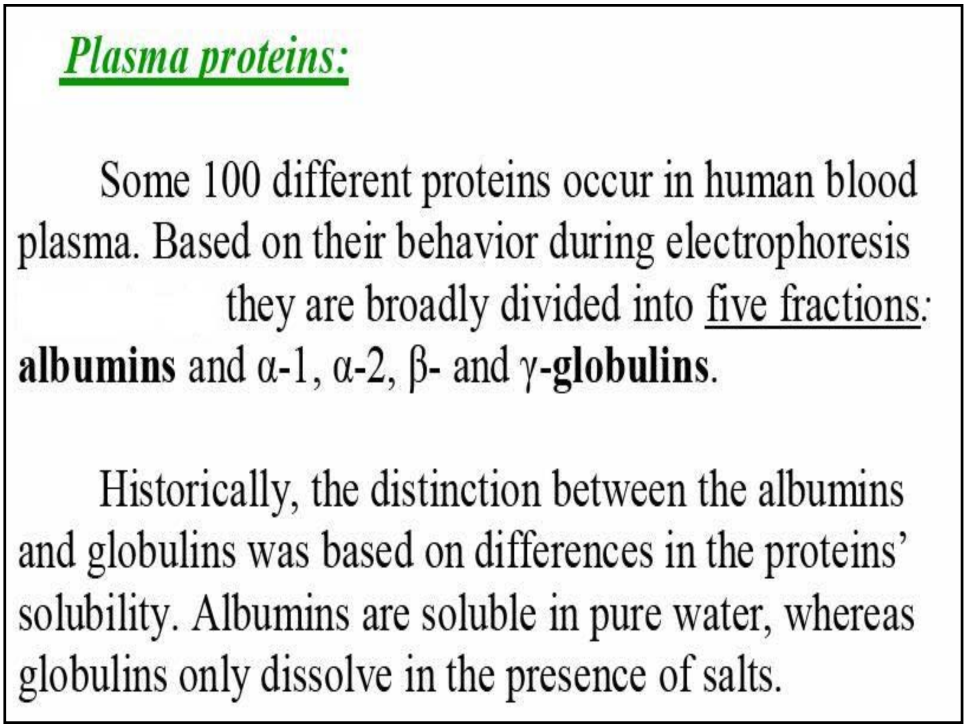
Plasma lipoproteins are separated into 4 bands when migrated in the electric field.





2-Plasma Proteins Electrophoresis

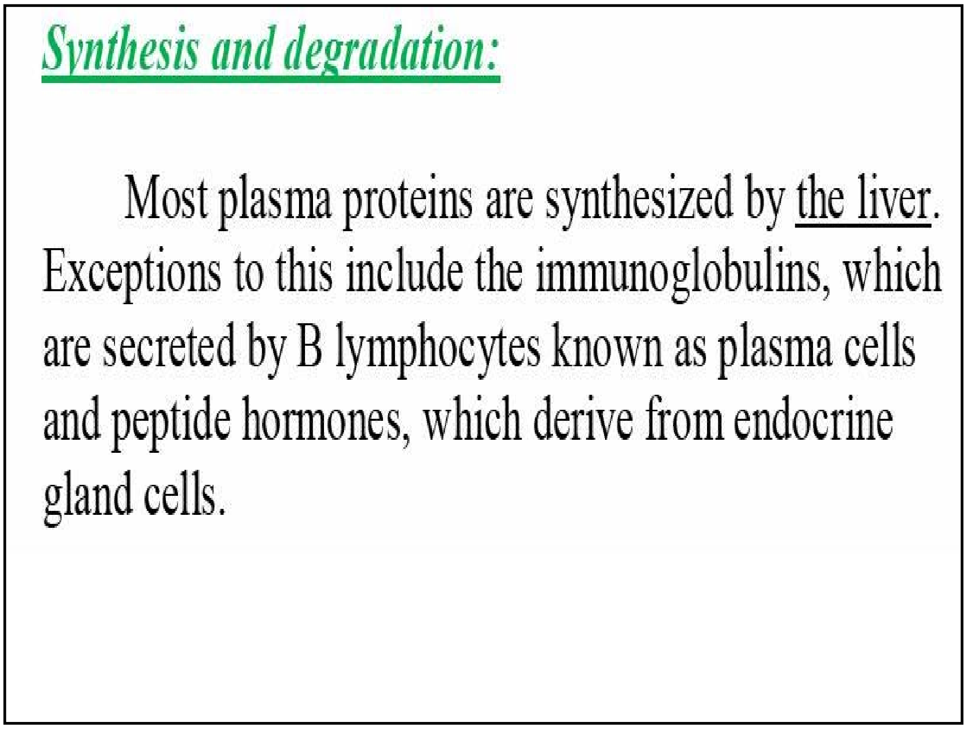
Quantitatively, proteins are the most important part of the soluble components of the blood plasma. With concentrations of between **60 and 80** g L^{-1}, they constitute approximately 4% of the body's total protein. Their tasks include transport, regulation of the water balance, hemostasis, and defense against pathogens.



Plasma proteins:

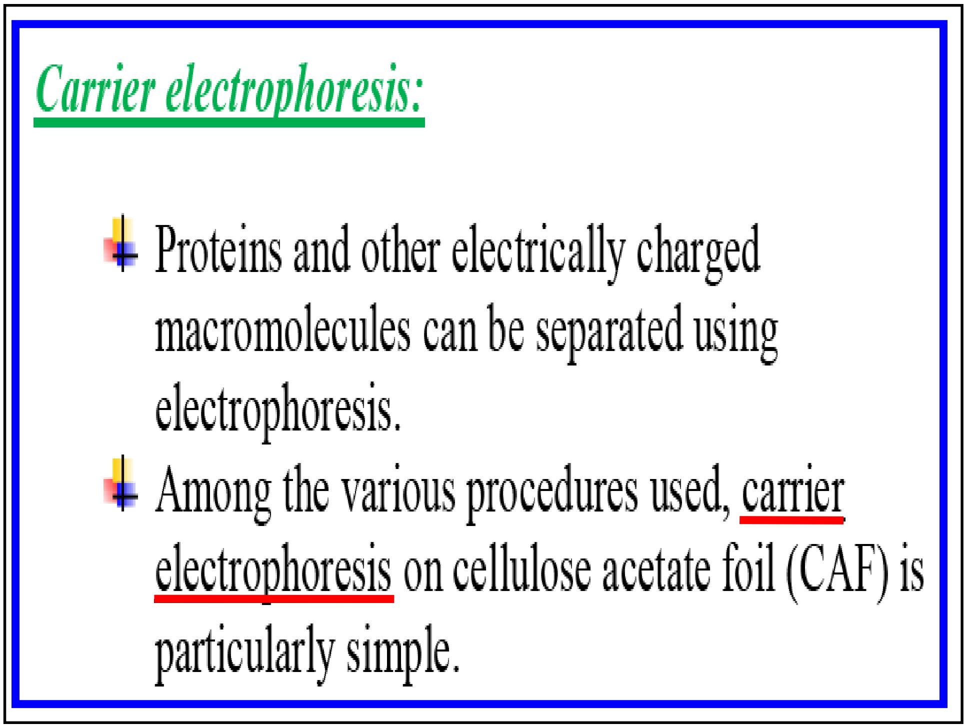
Some 100 different proteins occur in human blood plasma. Based on their behavior during electrophoresis they are broadly divided into five fractions: **albumins** and α-1, α-2, β- and γ-**globulins**.

Historically, the distinction between the albumins and globulins was based on differences in the proteins' solubility. Albumins are soluble in pure water, whereas globulins only dissolve in the presence of salts.



Synthesis and degradation:

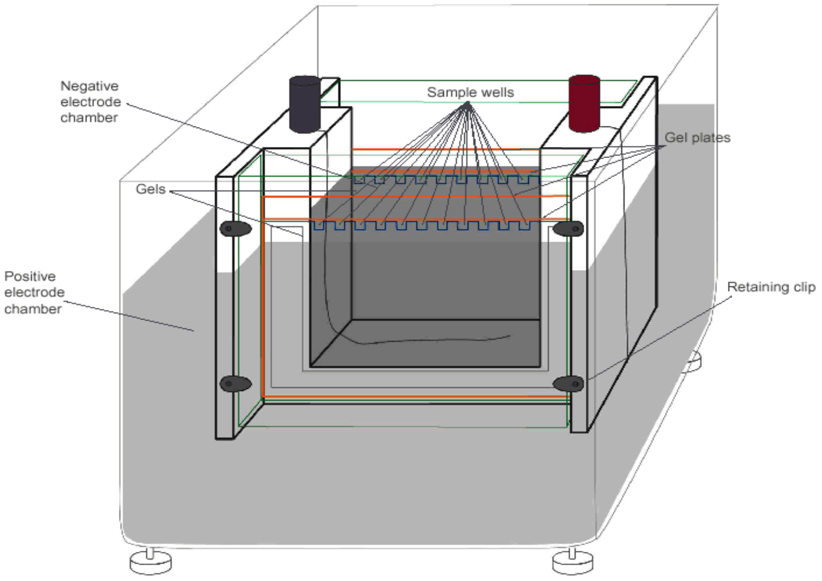
Most plasma proteins are synthesized by the liver. Exceptions to this include the immunoglobulins, which are secreted by B lymphocytes known as plasma cells and peptide hormones, which derive from endocrine gland cells.

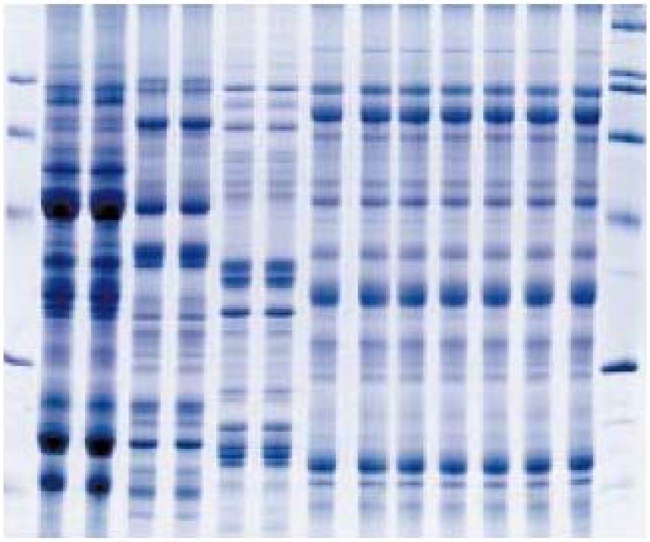


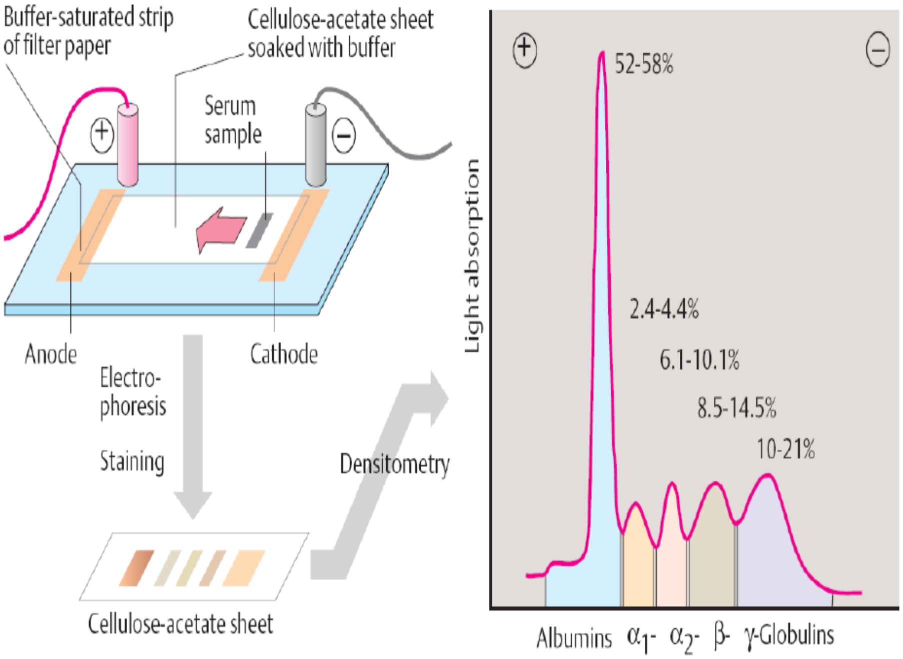
Carrier electrophoresis:

- Proteins and other electrically charged macromolecules can be separated using electrophoresis.
- Among the various procedures used, carrier electrophoresis on cellulose acetate foil (CAF) is particularly simple.

- Using this method, serum proteins which at slightly alkaline pH values all move towards the anode, due to their excess of negative charges can be separated into the five fractions mentioned.
- After the proteins have been stained with dyes, the resulting bands can be quantitatively assessed using densitometry.






Buffer-saturated strip of filter paper
Cellulose-acetate sheet soaked with buffer
Serum sample
+
−
Anode
Cathode
Electro-phoresis
Staining
Densitometry
Cellulose-acetate sheet
Light absorption
+
−
52-58%
2.4-4.4%
6.1-10.1%
8.5-14.5%
10-21%
Albumins
α1-
α2-
β-
γ-Globulins

Thanks